PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Attorney Docket No.: WPB 36400B

Date: August 24, 1998

Assistant Commissioner for Patents
Washington, D.C. 20231

BOX PATENT APPLICATION

CONTINUING APPLICATION TRANSMITTAL
RULE 1.53(b)

Sir:

Transmitted herewith for filing under 37 C.F.R. §1.53(b) is a

☐ Continuation ☒ Divisional ☐ Continuation-in-Part

application of prior pending Application No. 08/480,917, filed June 7, 1995,

For (Title): TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND TREATING CHAGAS DISEASE

By (Inventors): Glaucia PARANHOS-BACCALA; Mylene LESENECHAL and Michel JOLIVET

1. ☒ A Declaration and Power of Attorney is attached. The attached Declaration and Power of Attorney is:

   ☒ a. A copy of the Declaration and Power of Attorney from the parent application. (Used with the same or fewer inventors and (a) a copy of the prior application or (b) a revised, reformatted or edited version of the prior application that does not contain new matter.)

   ☐ b. A new Declaration and Power of Attorney. (Used with the same, fewer or additional inventors and (a) a copy of the prior application, (b) a revised, reformatted or edited version of the prior application that does not contain new matter, or (c) a new specification.)

2. ☒ The filing fee is calculated below:

CLAIMS IN THE APPLICATION AFTER ENTRY OF ANY PRELIMINARY AMENDMENT NOTED BELOW

FOR:	NO. FILED	NO. EXTRA
BASIC FEE		
TOTAL CLAIMS	20 - 20	= 0*
INDEP CLAIMS	1 - 3	= 0*
☐ MULTIPLE DEPENDENT CLAIMS PRESENTED		

* If the difference is less than zero, enter "0".

SMALL ENTITY			OTHER THAN A SMALL ENTITY	
RATE	FEE	OR	RATE	FEE
	$ 395	OR		$ 790
x 11 =	$	OR	x 22	$ -----
x 41 =	$	OR	x 82	$ -----
+135 =	$	OR	+270	$ -----
TOTAL	$	OR	TOTAL	$ 790

3. ☒ Check No. 61059 in the amount of $790.00 to cover the filing fee is attached. The Commissioner is hereby authorized to charge any other fees that may be required to complete this filing, or to credit any overpayment, to Deposit Account No. 15-0461. Two duplicate copies of this sheet are attached.

4. ☐ Cancel claims _____ of the application before calculating the filing fee. At least one independent claim is retained for filing purposes.

DEPOSIT ACCOUNT USE AUTHORIZATION
Please grant any extension necessary for entry;
Charge any fee due to our
Deposit Account No. 15-0461

5. ☒ Amend the specification by inserting before the first line the sentence:

--This is a ☐ Continuation ☒ Division ☐ Continuation-in-Part of Application No. 08/480,917 filed June 7, 1995. The entire disclosure of the prior application(s) is hereby incorporated by reference herein in its entirety.--

6. ☒ Formal drawings (Figs. 1-2) are attached.

7. ☒ Priority of foreign application No. 94 10132 filed August 12, 1994 in France is claimed under 35 U.S.C. §119 and/or §365(b).

☒ The certified copy was filed in prior Application No. 08/480,917 on September 7, 1995.

☐ A certified copy of the above foreign application(s) is filed herewith.

8. ☐ Priority of U.S. Provisional Application(s) No. _____ filed _____ is claimed under 35 U.S.C. §119.

☐ Amend the specification by inserting before the first line the sentence:

--This nonprovisional application claims the benefit of U.S. Provisional Application(s) No. _____ filed _____.--

9. ☒ The prior application is assigned of record to BIO MERIEUX recorded at Reel 7626, Frame 0182.

10. ☐ This application is filed by fewer than all the inventors named in the prior application (37 C.F.R §1.53(d)(4)). Delete the following inventor(s) named in the prior application:

_____ _____

_____ _____

11. ☒ A Preliminary Amendment is attached. Claims added by this Amendment are properly numbered consecutively beginning with the number next following the highest numbered claim in the application.

12. ☒ An Information Disclosure Statement is attached.

13. ☐ Small entity status:

☐ a. A small entity statement is attached.

☐ b. A small entity statement was filed in the parent application and such status is still proper and desired.

☐ c. Small entity status is no longer claimed.

14. ☐ Other: _____

15. ☒ The power of attorney in the application is to James A. Oliff, Registration No. 27,075, William P. Berridge, Registration No. 30,024, Kirk M. Hudson, Registration No. 27,562, Thomas J. Pardini, Registration No. 30,411, Edward P. Walker, Registration No. 31,450, Robert A. Miller, Registration No. 32,771 and/or Mario A. Costantino, Registration No. 33,565.

☒ a. The power appears in the attached Declaration and Power of Attorney.

☐ b. Since the power does not appear in the attached Declaration and Power of Attorney, a substitute Power of Attorney is also attached.

16. ☒ Address all future communications to:

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Respectfully submitted,

William P. Berridge
Registration No. 30,024

Melanie L. Mealy
Registration No. 40,085

WPB:CLC/jca

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Glaucia PARANHOS-BACCALA; Mylene LESENECHAL and Michel JOLIVET

Prior Group Art Unit: 1641

Application No.: Rule 1.53(b) Div. Appln. of U.S. Appln. No. 08/480,917, filed June 7, 1995

Prior Examiner: J. Shaver

Filed: August 24, 1998

Docket No.: WPB 36400B

For: TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND TREATING CHAGAS DISEASE

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D. C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE TITLE:

Page 1, above the first line, insert the following title: --TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND TREATING CHAGAS DISEASE.--

IN THE SPECIFICATION:

Please amend the specification as follows:

Page 1, before line 1, insert:

--FIELD OF THE INVENTION--;

between lines 5 and 6, insert:

--BACKGROUND OF THE INVENTION--.

Page 2, line 1, change "epi-mastigote" to --epimastigote--;

line 26, change "tests, such as" to --tests include--.

Page 3, line 29, change "where" to --were--.

Page 4, line 1, change "sero-logical" to --serological--;

line 4, change "anti-gen" to --antigen--;

between lines 6 and 7, insert:

--SUMMARY OF THE INVENTION--;

between lines 22 and 23, insert:

--DETAILED DESCRIPTION OF THE INVENTION--;

line 26, change "No.1," to --NO:1,--;

line 28, change "No.1," to --NO:1,--.

Page 5, line 5, change "No.1" to --NO:1--.

Page 7, line 10, change "No.1" to --NO:1,--;

line 12, change "No.1" to --NO:1, and--;

line 13, delete "and" (first occurrence);

line 14, after "2207" insert --of SEQ ID NO:1--;

line 26, change "No.2." to --NO:2.--.

Page 8, line 3, change "No.2," to --NO:2,--.

Page 9, line 4, change "(E" to --(E.--.

Page 10, line 7, change "No.2." to --NO:2.--.

Page 13, line 30, change "under-stood" to --understood--.

Page 15, line 19, change "No.1," to --NO:1,--;

line 24, change "No.1," to --NO:1,--.

Page 16, line 6, change "No.1." to --NO:1.--;

line 29, change "techniques," to --technique,--;

line 30, after "(14))," insert --the--;

line 32, before "Northern" insert --the--.

Page 17, line 2, after "target," insert --and the--;

line 4, change "tech-nique" to --technique--;

line 13, change "trans-lation" to --translation--;

line 14, change "pheno-mena." to --phenomena.--;

line 24, change "No.7," to --NO:7,--; change "No.8," to --NO:8,--; change "No.9," to --NO:9,--; change "No.10," to --NO:10, and--;

line 25, change "No.12." to --NO:12.--.

Page 18, between lines 12 and 13, insert:

--DESCRIPTION OF THE FIGURES--.

Page 19, line 8, change "No.3" to --NO:3--; change "24," to --24--;

line 10, change "No.4" to --NO:4--; change "24" to --24,--;

line 25, change "No.I" to --NO:1--;

line 26, change "No.2," to --NO:2,--;

line 28, change "1825." to --1825 of SEQ ID NO:1.--;

line 30, change "No.2." to --NO:2.--.

Page 20, line 23, change "For that, microtiter" to --Microtiter--;

line 24, change "name), nunc)" to --name))--;

line 30, delete "entire";

line 31, change "react" to --reacts--.

Page 21, line 31, change "Tc 50" to --Tc50--.

Page 22, line 17, change "No.5" to --NO:5--;

line 18, change "No.6" to --NO:6--;

line 20, change "No.5" to --NO:5--;

line 22, change "No.6" to --NO:6--;

line 26, change "No.1," to --NO:1,--.

Page 23, line 18, change "No.7" to --NO:7--;

line 19, change "No.8" to --NO:8--;

line 21, change "No.7" to --NO:7--;

line 25, change "No.8" to --NO:8--;

line 28, change "No.1," to --NO:1,--.

Page 24, line 3, change "No.1" to --NO:1--;

line 9, change "No.9 :" to --NO:9:--;

line 11, change "No.10:" to --NO:10:--;

line 13, change "No.9" to --NO:9--;

line 16, change "No.1." to --NO:1.--;

line 17, change "No.10" to --NO:10--;

line 20, change "No.10," to --NO:10--;

line 25, change "No.1" to --NO:1--.

Page 25, line 1, change "No.11" to --NO:11--;

line 7, change "No.12:" to --NO:12:--;

line 9, change "No.13:" to --NO:13:--;

line 11, change "No.12" to --NO:12--;

line 15, change "No.11" to --NO:11--;

line 17, change "No.11." to --NO:11.--;

line 19, change "des-cribed," to --described,--;

line 22, change "No.1" to --NO:1--.

Page 37, line 23, change "1426" to --426-428--.

Page 38, line 21, change "386-389" to --836-839--.

REMARKS

By this Preliminary Amendment, the specification has been amended to place the application in better condition for initial examination and allowance. Claims 1-20 are pending.

The computer readable form of the Sequence Listing in this application is identical with that filed in Application No. 08/480,917, filed on June 7, 1995. In accordance with 37 C.F.R. §1.821(e), please use the computer readable form filed in that application as the computer readable form for the instant application. It is understood that the U.S. Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application.

Amino acids 321-340 were inadvertently omitted from SEQ ID NO:2 on page 28 of the French-language patent application, filed June 7, 1995, and from SEQ ID NO:2 on page 32 of the Verified Translation. The amino acid sequence identified in SEQ ID NO:2

corresponds to the nucleotide sequence identified in SEQ ID NO:1. One of ordinary skill in the art would have recognized from these disclosures that a segment of amino acids was missing from SEQ ID NO:2 underneath identification numbers 321-340 and would have readily been able to determine the omitted amino acid segment by translating from the corresponding codons in SEQ ID NO:1. Thus, no new matter is added.

Early and favorable action on the merits is respectfully requested.

Respectfully submitted,

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The subject of the present invention is a new genetic material encoding a new protein recognized by anti-Trypanosoma cruzi antisera, and it relates to the use of said gene and protein, especially for diagnostic, pharmaceutical and therapeutic purposes.

Trypanosoma cruzi is a flagellate protozoal parasite, a member of the order Kinetoplastida and of the family Trypanosomatidae, which is responsible for Chagas disease which affects naturally millions of persons, mainly in Latin America.

In vertebrate hosts, Trypanosoma cruzi is present in two forms: one which is mobile by means of its flagellum or trypomastigote and which does not divide; the other is aflagellate, or intracellular amastigote, which multiplies by binary division.

Transmission of the protozoan in man occurs through hematophagous insects of the family Reduviidae, during a blood meal followed by dejections at the site of the bite. The vector insect thus releases the infectious metacyclic trypomastigote forms which will colonize many cell types through the blood circulation. Trypanosoma cruzi infects cardiac and skeletal muscular cells, the glial cells and the cells of the mononuclear phagocytic system. After passive penetration into the host cell, the trypomastigote form of the parasite differentiates into the amastigote form, divides actively and then this is followed by a release of the trypomastigote forms, thereby causing a new cell invasion.

The insects will complete the parasitic cycle by ingesting, during a blood meal, the trypomastigote forms in the host. The latter differentiate into

epi-mastigote forms in the vector's middle intestine and finally into the infectious metacyclic trypomastigote forms in the posterior intestine.

Two phases can be distinguished in the Chagas disease: the acute phase and the chronic phase. The acute phase occurs after a transfusional, congenital or vectorial type contamination and lasts for a few weeks. It is characterized by a large number of parasites circulating in the blood and corresponds to an exponential division of the protozoan. The acute phase is most often asymptomatic. However, in infants contaminated by their mother, the acute phase, which is marked by an acute cardiopathy, may be critical. The chronic phase may extend over many years. In some individuals, this phase is asymptomatic. On the other hand, other patients have tissue lesions in the heart or digestive type manifestations. In any case, clinical diagnosis must always be confirmed by tests for the detection either of antibodies directed against the parasitic antigens, or of the parasite itself.

This disease is becoming a worldwide problem because of the contamination through blood transfusion. It was therefore becoming essential to have available diagnostic tests which make it possible to determine the presence of the parasite in individuals. Various serological tests, such as direct agglutination, indirect immunofluorescence (IIF), complement fixation tests (CFR), ELISA tests (Enzyme Linked Immunosorbent Assay). The Trypanosoma cruzi antigens used for the serological tests are obtained from a total lysate of the noninfectious stage of the parasite or from partially purified protein fractions. However, these

fractions do not allow antigens to be obtained in sufficient quantity and quality for the production of a reliable serological diagnostic test. Furthermore, the complexity of the parasite and the strain-to-strain antigenic polymorphism introduce an additional difficulty in the reproducibility of the different preparations. Finally, there are many risks of cross-reactivity with other protozoa, more particularly with Trypanosoma rangeli, a nonpathogenic parasite, and the family Leishmania. Another disadvantage of these techniques is the absence of determination of the disease phase which would allow a treatment from the onset of the acute phase.

In order to solve these various problems, it was envisaged to produce a serological diagnostic kit composed of recombinant proteins which would be specific for Trypanosoma cruzi.

Various research groups have screened libraries for expression of Trypanosoma cruzi genomic DNA or complementary DNA in the vector λgt11, using sera from patients suffering from Chagas disease. The λgt11 phage allows the insertion of foreign DNA of a maximum size of 7Kb into the EcoR1 site localized in the lacZ gene, under the control of the lac promoter. The product obtained is a recombinant protein used with beta-galactosidase, which is inducible by IPTG (isopropyl beta-D-thiogalactoside).

Various Trypanosoma cruzi genes, encoding proteins recognized by the Chagasic sera where thus characterized. Among the recombinant antigens described, the H49 antigen may be mentioned (Paranhos et al., 1994 (1)). However, this antigen does not allow a

sero-logical detection sensitivity of 100% of the patients in the acute or chronic phase. It was therefore envisaged to combine the H49 antigen with the CRA anti-gen (Cytoplasmic Repetitive Antigen) (Lafaille et al., (1989) (2)) but still without solving this problem.

The present inventors have identified and obtained for the first time a new genetic material encoding a new protein, recognized by anti-Trypanosoma cruzi antisera, which makes it possible to overcome the abovementioned disadvantages. The genetic material may be used to produce proteins or polypeptides for the production of diagnostic tests, or for the preparation of vaccinal or pharmaceutical compositions, or may itself either be used as a probe, or for the determination of specific probes which can be used in nucleic acid hybridization tests for the detection of Trypanosoma cruzi infections. Likewise, the protein or any corresponding polypeptide may be used for the production of antibodies specific for the parasite, for diagnostic or passive protection purposes.

This gene was called Tc 100 by the applicant.

Consequently, the subject of the present invention is a DNA or RNA molecule consisting of at least one strand comprising a nucleotide sequence represented in the identifier SEQ ID No.1, or a sequence complementary or antisense or equivalent to said sequence identified in the identifier SEQ ID No.1, and especially a sequence having, for any succession of 100 contiguous monomers, at least 50%, preferably at least 60%, or better still at least 85% homology with said sequence.

Nucleotide sequence is understood to mean either a DNA strand or its complementary strand, or an RNA strand or its antisense strand or their corresponding complementary DNAs. The DNA sequence as represented in the identifier SEQ ID No.1 corresponds to the messenger RNA sequence, it being understood that the thymine (T) in the DNA is replaced by a uracil (U) in the RNA.

According to the invention, two nucleotide sequences are said to be equivalent in relation to each other, or in relation to a reference sequence if, functionally, the corresponding biopolymers can play essentially the same role, without being identical, with respect to the application or use considered, or in the technique in which they are involved; two sequences obtained because of the natural variability, especially spontaneous mutation, of the species from which they were identified, or because of induced variability, as well as homologous sequences, homology being defined below, are especially equivalent.

Variability is understood to mean any spontaneous or induced modification of a sequence, especially by substitution and/or insertion and/or deletion of nucleotides and/or of nucleotide fragments, and/or extension and/or shortening of the sequence at at least one of the ends; a nonnatural variability may result from the genetic engineering techniques used; this variability may result in modifications of any starting sequence, considered as reference, and capable of being expressed by a degree of homology relative to the said reference sequence.

Homology characterizes the degree of identity of two nucleotide (or peptide) fragments compared; it is measured by the percentage identity which is especially determined by direct comparison of nucleotide (or peptide) sequences, relative to reference nucleotide (or peptide) sequences.

Any nucleotide fragment is said to be equivalent to a reference fragment if it has a nucleotide sequence which is equivalent to the reference sequence; according to the preceding definition, the following are especially equivalent to a reference nucleotide fragment:

a) any fragment capable of at least partially hybridizing with the complementary strand of the reference fragment,

b) any fragment whose alignment with the reference fragment leads to the detection of identical contiguous bases, in greater number than with any other fragment obtained from another taxonomic group,

c) any fragment resulting or capable of resulting from the natural variability of the species, from which it is obtained,

d) any fragment capable of resulting from the genetic engineering techniques applied to the reference fragment,

e) any fragment, containing at least 30 contiguous nucleotides, encoding a peptide homologous or identical to the peptide encoded by the reference fragment,

f) any fragment different from the reference fragment by insertion, deletion, substitution of at least one monomer, extension or shortening at at least

one of its ends; for example any fragment corresponding to the reference fragment flanked at at least one of its ends by a nucleotide sequence not encoding a polypeptide.

The invention moreover relates to DNA or RNA fragments whose nucleotide sequence is identical, complementary, antisense or equivalent to any one of the following sequences:

- that starting at nucleotide 1232 and ending at nucleotide 2207 of SEQ ID No.1
- that starting at nucleotide 1232 and ending at nucleotide 1825 of SEQ ID No.1
- and that starting at nucleotide 1266 and ending at nucleotide 2207,

and especially the DNA or RNA fragments whose sequence has, for any succession of 30 contiguous monomers, at least 50%, preferably at least 60%, or better still at least 85% homology with any one of said sequences.

The subject of the invention is also a protein, called PTc100 by the applicant, having an apparent molecular mass of about 100 kDa, which is recognized by anti-Trypanosoma cruzi antisera, or an immunological equivalent of this protein, and fragments thereof. The amino acid sequence of this protein is represented in the identifier sequence SEQ ID No.2.

Immunological equivalent is understood to mean any polypeptide or peptide capable of being immunologically recognized by the antibodies directed against said Ptc100 protein.

The invention also relates to any fragment of the Ptc100 protein. A particular protein fragment has a

sequence starting at amino acid 323 and ending at amino acid 520 of the sequence defined in the identifier SEQ ID No.2, said fragment being specifically recognized by anti-Trypanosoma cruzi antisera; the invention also relates to any immunological equivalent of said fragment.

The Ptc100 protein and said protein fragments may contain modifications, especially chemical modifications, which do not alter their immunogenicity.

Moreover, the subject of the present invention is also an expression cassette which is functional especially in a cell derived from a prokaryotic or eukaryotic organism, and which allows the expression of DNA encoding the entire Ptc100 protein or a fragment thereof, in particular of a DNA fragment as defined above, placed under the control of elements necessary for its expression; said protein and said protein fragments being recognized by anti-Trypanosoma cruzi antisera.

Generally, any cell derived from a prokaryotic or eukaryotic organism can be used within the framework of the present invention. Such cells are known to persons skilled in the art. By way of examples, there may be mentioned cells derived from a eukaryotic organism, such as the cells derived from a mammal, especially CHO (Chinese Hamster Ovarian) cells; insect cells; cells derived from a fungus, especially a unicellular fungus or from a yeast, especially of the strain Pichia, Saccharomyces, Schizosaccharomyces and most particularly selected from the group consisting of Saccharomyces cerevisiae, Schizosaccharomyces pombe, Schizosaccharomyces malidevorans, Schizosaccharomyces

sloofiae, Schizosaccharomyces octosporus. Likewise, among the cells derived from a prokaryotic organism, there may be used, without this constituting a limitation, the cells of a strain of Escherichia coli (E coli) or enterobacterial cells. A large number of these cells are commercially available in collections, such as ATCC (Rockville, MA, USA) and AFRC (Agriculture & Food Research Council, Norfolk, UK). The cell may also be of the wild-type or mutant type. The mutations are described in the literature accessible to persons skilled in the art.

For the purposes of the present invention, an E. coli DH5a cell (marketed by the company CLONTECH under the reference: C2007-1) is used.

The expression cassette of the invention is intended for the production of the PTc100 protein or for fragments of said protein which are produced by the abovementioned E. coli cell, and which are recognized by human antisera. Such antisera are obtained from patients who have contracted a Trypanosoma cruzi infection recently or long ago, and contain immunoglobulins specifically recognizing PTc100. Of course, the PTc100 protein can also be recognized by other antibodies, such as for example monoclonal or polyclonal antibodies obtained by immunization of various species with the natural abovementioned protein, the recombinant protein or fragments or peptides thereof.

PTc100 protein is understood to mean the natural Trypanosoma cruzi cytoplasmic antigen, or the antigen produced especially by the genetic recombination techniques described in the present application, or any fragment or mutant of this antigen, provided

that it is immunologically reactive with antibodies directed against the PTc100 protein of this parasite.

Advantageously, such a protein has an amino acid sequence having a degree of homology of at least 70%, preferably of at least 85%, and most preferably of at least 95% relative to the sequence identified in the identifier SEQ ID No.2. In practice, such an equivalent can be obtained by deletion, substitution and/or addition of one or more amino acids of the native or recombinant protein. It is within the capability of persons skilled in the art to perform, using known techniques, these modifications without affecting immunological recognition.

Within the framework of the present invention, the PTc100 protein can be modified in vitro, especially by deletion or addition of chemical groups, such as phosphates, sugars or myristic acids, so as to enhance its stability or the presentation of one or several epitopes.

The expression cassette according to the invention allows the production of a PTc100 protein (having an amino acid sequence as specified above) and fragments of said protein, fused with an exogenous element which can help its stability, its purification, its production or its recognition. The choice of such an exogenous element is within the capability of persons skilled in the art. It may be especially a hapten, an exogenous peptide or a protein.

The expression cassette according to the invention comprises the elements necessary for the expression of said DNA fragment in the cell considered. "Elements necessary for the expression" is understood

to mean the elements as a whole which allow the transcription of the DNA fragment into messenger RNA (mRNA) and the translation of the latter into protein.

The present invention also extends to a vector comprising an expression cassette according to the invention. This may be a viral vector and especially a vector derived from a baculovirus, more particularly intended for expression in insect cells, or an adenovirus-derived vector for expression in mammalian cells.

It may also be an autonomously replicating plasmid vector and in particular a multiplicative vector.

The present invention also relates to a cell derived from a prokaryotic or eukaryotic organism, comprising an expression cassette, either in a form integrated in the cellular genome, or inserted in a vector. Such a cell was previously defined.

The subject of the present invention is also a process for preparing a PTc100 protein, or fragments of said protein, according to which:

(i) a cell derived from a prokaryotic or eukaryotic organism, comprising the expression cassette according to the invention, is cultured under appropriate conditions; and

(ii) the expressed protein derived from the abovementioned organism is recovered.

The present invention also relates to one or more peptides, whose amino acid sequence corresponds to a portion of the sequence of the PTc100 protein and exhibiting, alone or as a mixture, a reactivity with the entire sera from individuals or animals infected with Trypanosoma cruzi.

The peptides can be obtained by chemical synthesis, lysies of the PTc100 protein or by genetic recombination techniques.

The invention also relates to monoclonal or polyclonal antibodies obtained by immunological reaction of a human or animal organism to an immunogenic agent consisting of the natural or recombinant PTc100 protein and fragments thereof, or of a peptide, as defined above.

The present invention also relates to a reagent for the detection and/or monitoring of a Trypanosoma cruzi infection, which comprises, as reactive substance, a PTc100 protein as defined above, or fragments thereof, a peptide or a mixture of peptides as defined above, or at least one monoclonal or polyclonal antibody as described above.

The above reagent may be attached directly or indirectly to an appropriate solid support. The solid support may be especially in the form of a cone, a tube, a well, a bead and the like.

The term "solid support" as used here includes all materials on which a reagent can be immobilized for use in diagnostic tests. Natural or synthetic materials, chemically modified or otherwise, can be used as solid supports, especially polysaccharides such as cellulose-based materials, for example paper, cellulose derivatives such as cellulose acetate and nitrocellulose; polymers such as vinyl chloride, polyethylene, polystyrenes, polyacrylate or copolymers such as polymers of vinyl chloride and propylene, polymers of vinyl chloride and vinyl acetate; styrene-based copoly-

mers, natural fibers such as cotton and synthetic fibers such as nylon.

Preferably, the solid support is a polystyrene polymer or a butadiene/styrene copolymer. Advantageously, the support is a polystyrene or a styrene-based copolymer comprising between about 10 and 90% by weight of styrene units.

The binding of the reagent onto the solid support may be performed in a direct or indirect manner.

Using the direct manner, two approaches are possible: either by adsorption of the reagent onto the solid support, that is to say by noncovalent bonds (principally of the hydrogen, Van der Walls or ionic type), or by formation of covalent bonds between the reagent and the support. Using the indirect manner, an "anti-reagent" compound capable of interacting with the reagent so as to immobilize the whole onto the solid support can be attached beforehand (by adsorption or covalent bonding) onto the solid support. By way of example, there may be mentioned an anti-PTc100 antibody, on the condition that it is immunologically reactive with a portion of the protein different from that involved in the reaction for recognizing the antibodies in the sera; a ligand-receptor system, for example by grafting onto the PTc100 protein a molecule such as a vitamin, and by immobilizing onto the solid phase the corresponding receptor (for example the biotin-streptavidin system). Indirect manner is also under-stood to mean the preliminary grafting or fusion by genetic recombination of a protein, or a fragment of this protein, or of a polypeptide, to one end of the

PTc100 protein, and the immobilization of the latter onto the solid support by passive adsorption or covalent bonding of the protein or of the polypeptide grafted or fused.

The invention also relates to a process for the detection and/or monitoring of a Trypanosoma cruzi infection in a biological sample, such as a blood sample from an individual or an animal likely to have been infected with Trypanosoma cruzi, characterized in that said sample and a reagent as defined above are placed in contact, under conditions allowing a possible immunological reaction, and the presence of an immune complex with said reagent is then detected.

By way of non-limiting example, there may be mentioned the sandwich-type detection process in one or more stages, as especially described in patents FR 2,481,318 and FR 2,487,983, which consists in reacting a first monoclonal or polyclonal antibody specific for a desired antigen, attached onto a solid support, with the sample, and in revealing the possible presence of an immune complex thus formed using a second antibody labelled by any appropriate marker known to persons skilled in the art, especially a radioactive isotope, an enzyme, for example peroxidase or alkaline phosphatase and the like, using so-called competition techniques well known to persons skilled in the art.

The subject of the invention is also an active immunotherapeutic composition, especially a vaccinal preparation, which comprises as active ingredient, a natural or recombinant PTc100 protein or fragments thereof, or the peptides identified above, the active ingredient being optionally conjugated with a pharma-

ceutically acceptable carrier, and optionally an excipient and/or an appropriate adjuvant.

The present invention also covers a pharmaceutical composition intended for the treatment or for the prevention of a Trypanosoma cruzi infection in man or in an animal, comprising a therapeutically effective quantity of an expression cassette, a vector, a cell derived from a prokaryotic or eukaryotic organism as defined above, a PTc100 protein according to the invention, or fragments thereof, or an antibody of the invention.

The subject of the present invention is also probes and primers specific for T. cruzi, and their uses in diagnostic tests.

The term probe as used in the present invention refers to a DNA or RNA containing at least one strand having a nucleotide sequence which allows hybridization to nucleic acids having a nucleotide sequence as represented in the identifier SEQ ID No.1, or a complementary or antisense sequence, or a sequence equivalent to said sequence, and especially a sequence having, for any succession of 5 to 100 contiguous monomers, at least 50%, preferably at least 60%, or even better at least 85% homology with SEQ ID No.1, with fragments thereof, or with a synthetic oligonucleotide allowing such a hybridization, nonmodified or comprising one or more modified bases such as inosine, 5-methyldeoxycytidine, deoxyuridine, 5-dimethylaminodeoxyuridine, 2,6-diaminopurine, 5-bromodeoxyuridine or any other modified base. Likewise, these probes may be modified at the level of the sugar, namely the replacement of at least one deoxyribose with a polyamide (P. E. NIELSEN

et al. (1991) (13)), or at the level of the phosphate group, for example its replacement with esters, especially chosen from esters of diphosphate, of alkyl and arylphosphonate and of phosphorothioate.

The probes may be much shorter than the sequence identified in the identifier SEQ ID No.1. In practice, such probes comprise at least 5 monomers, advantageously from 8 to 50 monomers, having a hybridization specificity, under defined conditions, to form a hybridization complex with DNA or RNA having a nucleotide sequence as defined above.

A probe according to the invention can be used for diagnostic purposes as capture and/or detection probe, or for therapeutic purposes.

The capture probe can be immobilized on a solid support by any appropriate means, that is to say directly or indirectly, for example by covalent bonding or passive adsorption.

The detection probe is labelled by means of a marker chosen from radioactive isotopes, enzymes especially chosen from peroxidase and alkaline phosphatase, and those capable of hydrolyzing a chromogenic, fluorigenic or luminescent substrate, chromophoric chemical compounds, chromogenic, fluorigenic or luminescent compounds, nucleotide base analogs, and biotin.

The probes of the present invention which are used for diagnostic purposes can be used in any known hybridization techniques, and especially the so-called "Dot-Blot" technique (Maniatis et al. (1982) (14)), Southern Blotting technique (Southern E. M. (1975) (15)), Northern Blotting technique, which is a

technique identical to the Southern Blotting technique but which uses RNA as target, sandwich technique (Dunn A.R. et al. (1977) (16)). Advantageously, the sandwich tech-nique is used which comprises a specific capture probe and/or a specific detection probe, it being understood that the capture probe and the detection probe must have a nucleotide sequence which is at least partially different.

Another application of the invention is a therapeutic probe for treating infections due to Trypanosoma cruzi, said probe being capable of hybridizing in vivo with the DNA or RNA of the parasite to block the trans-lation and/or transcription and/or replication pheno-mena.

A primer is a probe comprising 5 to 30 mono-mers, having a hybridization specificity, under prede-fined conditions, for the initiation of an enzymatic polymerization, for example in an amplification tech-nique such as PCR (Polymerase Chain Reaction), in an elongation process such as sequencing, in a reverse transcription method and the like.

A preferred probe or primer will contain a nucleotide sequence chosen from the sequences SEQ ID No.7, SEQ ID No.8, SEQ ID No.9, SEQ ID No.10, SEQ ID No.12.

The invention also relates to a reagent for detecting and/or identifying Trypanosoma cruzi in a biological sample, comprising at least one probe as defined above, and in particular a capture probe and a detection probe, either or both corresponding to the above definition.

The invention therefore provides a process for selectively detecting and/or for identifying Trypanosoma cruzi in a biological sample, according to which the RNA, extracted from the parasite and optionally denatured, or the DNA, denatured extract, or the DNA obtained from reverse transcription of the RNA, is exposed to at least one probe as defined above and the hybridization of said probe is detected.

The invention will be understood more clearly upon reading the detailed description below which is made with reference to the accompanying figures in which:

Figure 1 represents the restriction map of the Tc100 gene, which map is deduced by Southern blotting of different fragments obtained after digestion of Trypanosoma cruzi DNA with restriction endonucleases.

Figure 2 is a schematic representation of the three overlapping regions of the Tc100 cDNA. The numbered arrows represent the oligonucleotides used as primers for the PCR amplification.

Example 1: Isolation of the Tc50 clone

An expression library was constructed from Trypanosoma cruzi genomic DNA fragments. The T. cruzi, strain G (YOSHIDA. N, (1983) (17)), DNA isolated from the metacyclic trypomastigote stage was digested with the enzyme DNase I. After selection of the fragments according to their size, they were ligated to synthetic EcoRI adaptors and cloned into the EcoRI site of lambda gt11 vector DNA (Young and Davis, 1983 (3); Cotrim et al., 1990) (4).

The clone, called Tc50 by the applicant, was isolated from the library by immunological screening with the aid of a mixture of sera from patients suffering from the chronic phase of the Chagas disease.

The Tc50 phage clone was purified, amplified and the insert was detected by the PCR ("Polymerase Chain Reaction") technique with the aid of the primers:

SEQ ID No.3 5'(GGTGGCGACGACTCCTGGAGCCCG)3' 24,

and

SEQ ID No.4 5'(TTGACACCAGACCAACTGGTAATG)3' 24

corresponding respectively to the nucleotide sequence of the left and right arms of the lambda gt11 phage DNA.

The 594 base pairs (bp) Tc50 DNA fragment, after EcoRI digestion, was subcloned into the expression vector pGEX (Pharmacia) linearized with EcoRI. The sequencing of the Tc50 clone DNA was carried out in this same vector with the aid of specific primers situated in 3' and 5' of the cloning site of pGEX, according to the chain termination technique (Sanger et al., 1977 (5)) and according to the manufacturer's procedure (USB-Amersham).

The nucleotide sequence of the 594 bp Tc50 fragment as well as its deduced amino acid sequence (198 aa) are represented in the identifiers SEQ ID No.I and SEQ ID No.2, respectively. The nucleotide sequence of the 594 bp Tc50 fragment starts at nucleotide (nt) 1232 and ends at nucleotide 1825. The corresponding amino acid sequence starts at amino acid 323 and ends at amino acid 520 of SEQ ID No.2.

Example 2: Expression of the Tc50 clone in Escherichia coli

The construct pGEX-Tc50 (198 aa) synthesizes, in the bacterium DH5alpha, a protein fused with GST ("Glutathione S Transferase"), with an apparent molecular mass of 50 kDa, which is detected by SDS-PAGE polyacrylamide gel electrophoresis (SDS: sodium dodecyl sulfate) (Laemmli, 1970 (6)). The reactivity of the protein towards chagasic human sera was confirmed by the Western blotting technique (Towbin et al., 1979 (7)) with the aid of the same mixture of chronic phase chagasic sera which is used for screening the lambda gt11 library.

The soluble fraction of the recombinant GST-Tc50 protein obtained after lysis of the bacterial extracts by ultrasound was purified by affinity chromatography on a glutathione agarose column (Sigma), according to the method of Smith and Johnson, (1988) (8).

The antigenic properties of the recombinant GST-Tc50 antigen were tested by ELISA (Voller et al., 1975 (9)). For that, microtiter plates (Maxisorp (trade name), nunc) were sensitized with 100 ng/ml of GST-Tc50 antigen in 100 mM $NaHCO_3$ (pH 9.6). After incubation with the patients' sera, the immune complexes were detected with the aid of a peroxidase-coupled anti-human IgG goat serum.

The results are presented in the accompanying table and show that the entire chagasic human sera tested react specifically with the recombinant protein. No cross-reactivity was observed on 7 sera from

patients suffering from cutaneous or visceral leishmaniosis.

Example 3: Identification of the native T. cruzi protein having the antigenic determinants of the Tc50 clone

The detection of the native T. cruzi protein was performed after immunopurification of a mixture of chagasic human sera on the corresponding recombinant protein called PTc50 by the applicant.

The eluate of monospecific polyclonal antibodies which is obtained was used as probe, in Western blotting, on total protein extracts of different stages of the parasite. The selected antibodies specifically reacted with a protein of apparent molecular mass 100 kDa, called PTc100 by the applicant, which is expressed in all the tested strains of the parasite.

Example 4: Molecular analysis of the Tc100 gene -Southern blots

In order to establish the restriction map of the Tc100 gene (Figure 1), the T. cruzi, strain G, nuclear DNA was digested with different restriction endonucleases (BamHI, EcoRI, HindIII, PstI, PvuII, SacI, BamHI/EcoRI, BamHI/PvuII, EcoRI/HindIII, EcoRI/PstI, EcoRI/PvuII, EcoRI/SacI, PstI/SacI, PstI/PvuII, PvuII/SacI, PvuII/HindIII), separated on agarose gel and then transferred onto a nylon filter according to the Southern technique. The Southern blot hybridization was performed with the 594 bp Tc 50 DNA, which is a fragment of the Tc100 DNA described above,

radiolabelled with ^{32}P by random incorporation (Amersham).

- Cloning of a 3500 bp Tc100 genomic fragment

According to the results obtained by Southern blotting, the T. cruzi, strain G, genomic DNA was digested with the enzyme EcoRI and then separated on agarose gel. The EcoRI restriction fragments of about 3500 bp (Figure 1) were cloned into the vector lambda gt10 (Huynh et al., 1984 (10)) linearized by EcoRI. The phage clone containing the 3500 bp Tc100 genomic insert was isolated with the aid of the 594 bp radiolabelled probe described above. A 1041 bp fragment situated in the 3' region of the 3500 bp Tc100 genomic insert was sequenced. This sequencing was carried out gradually with the aid of the following primers:

SEQ ID No.5 5' (TCGGGCACTGACGCGGCG) 3' 18

SEQ ID No.6 5' (CTTATGAGTATTTCTTCCAGGGTA) 3' 24

The primer SEQ ID No.5 is situated in the previously sequenced portion of the 594 bp Tc50 fragment. The primer SEQ ID No.6 corresponds to the lambda gt10 phage primer.

This 1041 bp fragment, which starts at nucleotide 1403 and ends at nucleotide 2443 of SEQ ID No.1, has an open reading frame in phase with the sequence of the 594 bp Tc50 fragment.

Example 5: Cloning of the Tc100 cDNA

The cDNA was synthesized from total RNA from T. cruzi, strain G, epimastigots. The Tc100 cDNA was amplified by the PCR technique in three different fragments: a fragment A corresponding to the 5' region of 1459 bp, a fragment B corresponding to the central region of 942 bp, a fragment C corresponding to the 3' region of 1406 bp of the Tc100 cDNA, as schematically represented in Figure 2.

- Cloning of fragment A of the Tc100 cDNA

The total cDNA synthesized by AMV ("avian myeloblastosis virus") reverse transcriptase, with the aid of random hexanucleotides (Boehringer Mannheim), was amplified, by PCR, using the following pair of primers:

SEQ ID No.7 5'(AACGCTATTATTAGAACAGTT)3' 21, and

SEQ ID No.8 5'(TGCAGCAGCGGCAGAAGT)3' 18

SEQ ID No.7 corresponds to a portion of the consensus sequence of 35 nucleotides present in 5' of the mRNAs in trypanosomatides and called "spliced leader" (Parsons et al. 1984 (11)).

SEQ ID No.8 corresponds to the sequence complementary to a portion of the predetermined sequence of the 594 bp fragment, and starts at nucleotide 1442 and ends at nucleotide 1459 of SEQ ID No.1, according to the coding strand numbering.

After verification by Southern blotting with the aid of the radiolabelled 594 bp probe previously described, the 1459 bp cDNA fragment corresponding to

the 5′ region of Tc100 was cloned into the plasmid called pCRII (trade name) (Invitrogen), and sequenced. The sequence represented in the identifier SEQ ID No.1 starts at nucleotide 1 and ends at nucleotide 1459.

- Cloning of fragment B of the Tc100 cDNA

The T. cruzi total cDNA was amplified by PCR with the aid of the primers:

SEQ ID No.9 : 5′(CAGCCGACGGTAGCTGCGTCCT)3′ 22

and

SEQ ID No.10: 5′(ACATAATGGCCTCGTTCACAC)3′ 21

The sequence ID No.9 which corresponds to a portion of the 594 bp predetermined sequence of the Tc100 gene starts at nucleotide 1266 and ends at nucleotide 1287 of SEQ ID No.1.

The sequence SEQ ID No.10 corresponds to the sequence complementary to a portion of the 1041 bp previously described sequence of the Tc100 gene. This sequence SEQ ID No.10 starts at nucleotide 2187 and ends at nucleotide 2207 of SEQ ID No.1, according to the coding strand numbering.

The fragment obtained, 942 bp in length, was cloned into the plasmid pCRII and sequenced. The sequence represented in the identifier SEQ ID No.1 starts at nucleotide 1266 and ends at nucleotide 2207.

- Cloning of fragment C of the Tc100 cDNA

In order to isolate the 3′ portion of the Tc100 cDNA, the T. cruzi total cDNA was synthesized with the aid of the adaptor oligo$(dT)_{16}$ hybrid primer.

SEQ ID No.11 5'(GACTCGCTGCAGATCGATTTTTTTTTTTTTTTT)3' 34 according to the RACE ("Rapid Amplification of cDNA Ends") procedure (Frohman et., 1988 (12)).

The 3' region of the Tc100 cDNA was amplified using the adaptor primer and the following pair of primers:

SEQ ID No.12: 5'(CGAAGAGACCATGAACAACTT)3' 21

and

SEQ ID No.13: 5'(GACTCGCTGCAGATCGAT)3' 18

The sequence SEQ ID No.12 corresponds to a portion of the previously described 1041 bp sequence of the Tc100 gene, starting at nucleotide 1997 and ending at nucleotide 2017.

The sequence SEQ ID No.11 corresponds to the arbitrary sequence of the adaptor represented in SEQ ID No.11.

After checking by Southern blotting using the 1041 bp radiolabelled fragment previously des-cribed, the 3' fragment of the Tc100 cDNA, 1423 bp long, was cloned into pCRII and sequenced. The sequence represented in the identifier SEQ ID No.1 starts at nucleotide 1997 and ends at nucleotide 3402.

The Tc100 complete cDNA, 3402 bp in size, was completely sequenced. It has a 2745 bp open reading frame and the deduced amino acid sequence is 915. The methionine codon is in position 266 and the stop codon in position 3011.

The Trypanosoma cruzi Tc100 gene encodes the new PTc100 protein of theoretical molecular mass 100 kDa.

Of course, since the DNA sequence of the gene has been fully identified, it is possible to produce the corresponding DNA solely by chemical synthesis, and then to insert the DNA into commercially available DNA vectors, using known techniques from the technology relating to genetic recombination.

TABLE

Disease	Sera	OD (492nm) detection threshold = 0.320
CHAGAS DISEASE	1	1.358 (+)
	2	1.278 (+)
	3	0.328 (+)
	4	0.404 (+)
	5	1.378 (+)
	6	1.059 (+)
	7	0.895 (+)
	8	1.791 (+)
	9	1.635 (+)
	10	1.427 (+)
	11	1.009 (+)
	12	1.743 (+)
	13	0.530 (+)
	14	1.035 (+)
	15	0.461 (+)
CUTANEOUS LEISHMANIOSIS	16	0.291 (-)
VISCERAL LEISHMANIOSIS (Kala azar)	17	0.071 (-)
	18	0.081 (-)
	19	0.279 (-)
	20	0.098 (-)
	21	0.067 (-)
	22	0.125 (-)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: PARANHOS-BACCALA, Glaucia
LESENECHAL, Mylene
JOLIVET, Michel

(ii) TITLE OF INVENTION: NEW TRYPANOSOMA CRUZI ANTIGEN, AND GENE ENCODING THE LATTER; THEIR APPLICATION TO THE DETECTION OF CHAGAS DISEASE

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Oliff & Berridge
(B) STREET: 700 South Washington Street, Suite 300
(C) CITY: Alexandria
(D) STATE: Virginia
(E) COUNTRY: U.S.A.
(F) ZIP: 22314

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/480,917
(B) FILING DATE: 07-JUN-1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Berridge, William P.
(B) REGISTRATION NUMBER: 30,024
(C) REFERENCE/DOCKET NUMBER: WPB 36400

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 703-836-6400
(B) TELEFAX: 703-836-2787

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3402 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AACGCTATTA TTAGAACAGT TTCTGTACTA TATTGTCATT TGGGGAGGGG GGAAAGGGGG 60

GAAGTACTTG CCGTTTTGTG TGGGTGACGA GACAACACAC ATCGAGCGGG AAGAAAAAAA 120

AAAAGGAAAT AAATTAAATT AAATTATTTG TTCTTTGAAT AGGCAAAGAA GAAGAAGAAG 180

AAAAGGTGCG GGGGAGGGAG GAGAAAGCGA CACACACACA AAAAAAAAAA AAGGAATTGC 240

GGAAATAACA ACGCAAGGCG CGGACATGAC CGTGACGGTG GATTTGTTCA ATCATGCGAA 300

GCCGAGCAAC AATGAGGGCC GCGTGTGGTC TGTGGACGCC GCGACATTTA ACGAGGTGCC 360

TGAGGCGCAG CGTGTGCTGG CGGATTCGCA GTTTTATCTT GCCTACACCA TGAAGCGGCG 420

TCACGTGCTG CGTGTGGTGA AGCGCTCGAA CCTTTTGAAG GGCACCGTGC GGGCACACTC 480

AAAGCCCATT CATGCGGTGA AGTTTGTGAA TTACCGCAGT AACGTCGCAG CATCGGCTGG 540

GAAGGGGGAG TTCTTCGTGT GGGTTGTGAC GGATGAAACG GAGGCGAGCA ACGGCAAGCC 600

GGATCTCGCA GCCCGCCTCA CAGTGAAGGT GTACTTTAAG CTTCAGGATC CTGTCACAAT 660

TCCATGCTTT TCTTTCTTTA TCAACGCCGA GAGTCAGCGG CCTGATCTGC TTGTCCTTTA 720

CGAAACGCAG GCGGCAATTC TTGACAGCTC CTCCCTCATT GAGCGCTTTG ACGTGGAATC 780

ACTGGAGGCA ACACTACAGC GGAATTGCAC AACCCTGCGA ACCCTGACTC AACCGGTTAG 840

TGAGAACAGT TTATGCTCCG TTGGCTCTGG CGGATGGTTC ACCTTTACCA CGGAACCAAC 900

AATGGTAGCG GCATGCACAT TACGAAACCG CAGCACTCCA TCATGGGCGT GTTGCGAGGG 960

TGAGCCAGTG AAGGCATTGC ATCTCCTTGA CGCAACCGTT GAGGAAAATG TCAGTGTTCT 1020

CGTGGCCGCA TCTACAAAAG GGGTGTACCA ATGGCTCCTT ACGGGTGTAG CAGAACCAAA 1080

CTTGTTGCGC AAGTTTGTCA TTGATGGATC TATTGTCGCG ATGGAAAGCT CACGAGAAAC 1140

GTTTGCCGTG TTTGACGACA GGAAGCAGCT GGCGCTGGTC AACATGCATT CCCCTCATAA 1200

CTTTACCTGC ACACACTACA TGATGCCTTG TCAGGTACAG CGTAACGGCT TTTGCTTCAA 1260

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GACTTCTGCC GCTGCTGCAT CCCCTGCATC ACCCCCTGTT TCAGCGCCAG CCAAGGCAGC 1500

CGCGCCTCCT GCCGCGGCGC GATCGGCTGA GCCGCACGTG GGGAGCAAGA TCATTGCTAA 1560

TCTAGTGAAT CAGCTGGGGA TTAATGTCAC CCAAAGGAGC GTCGTCAGCA CTGGAGCGCC 1620

GGCCACGACG AGGTCTACGG CGGTGACGTC CACGACTACC GCCCCGCAGC GAACAAGTCC 1680

ATACGGGCAC AATGGCCGAC CTGTGACGGC TGGATTGGTG GCAGCTAATA GTGGTGCCAG 1740

CGCGGCCTCG TCTCCCACAG CCGCGGCGAA ACCAACAGGA GAAGAAAAGG CCTCCGCGGC 1800

ATGTGAAACG AGCTCCGTGG CGATAAATGC GACACGCCCG GCGCTTCACA ACGCCTCTCT 1860

CCCGCAGGCG CCAACGGATG GCGTTTTGGC GGCAGCAGTA TACCAGTCGG AGGGCGAGGT 1920

TCATCAGTCG CTGGAGCGGC TGGAGTCCGT CATAACCAAC ACGTCTCGGG TTCTGAAGTT 1980

GCTCCCTGAC ACCATTCGAA GAGACCATGA ACAACTTCTG AATCTGGGTT TAGAGGCACA 2040

GATGACAGAG CTGCAGCAGA GCCGTCCAAC ACCGCAAACA CAGCCGAGAG ACACAAGCTC 2100

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CAACATCACG AAGGGGGTGA AGCGTGGTGT GAACGAGGCC ATTATGTTGC ATCTCGACCA 2220

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CACTTCTCTC GTGAAGGAAA ATGCCTCATT ACAGAAAGAG TTGAATTCCA TAATGTCTAG 2460

TGGGGTGTTG GATGAAATGC GTCGTATGCG GGAAGAGCTG TGCACATTGC GAGAGTCCGT 2520

TGCGAAGCGG AAGGCAACAA TGCCAGATTC TTCTCTTCAC GCCACGAGCT CCTTTCAAGG 2580

AAGAAGGTCT GCGCCCGAGA CAATTCTTGC AACCGCGTTA TCGATGGTGC GAGAGCAGCA 2640

ATACCGTCAG GGACTGGAAT ACATGTTGAT GGCTCAGCAG CCCTCTCTCC TCCTGCGGTT 2700

CCTCAGCATA CTTACAAGGG AAAACGAAAA CGCCTACTCG GAACTTATTG AAAATGTAGA 2760

GACGCCGAAT GACGTGTGGT GTTCGGTTCT GTTGCAACTC ATAGAGGCCG CGGCGACCGA 2820

GGCTGAGAAG GAGGTGGTTG TTGGCGTCGC CATTGATATT CTCTCCGAGC GCGATCAAAT 2880

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GGCAAGGTCG GAGACAACGA GCAGGTCATT CTTGCAATGC CTGAAGAACC TGGAAAAGCT 3000

TCTGCAATCA TGATAATAAA AAGAACTCAA CGAATACAGT TGTTGATTAT TAAGGAAGGG 3060

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GAAAACGTGT GGATAAGAAG GAGGGGTTTT GTGTGCGACC AGGAATTACT GGGGAACGCT 3180

GCTACACGGC GGAATCGACC ATTTTATTAT TATTATTATT GTCTTTAGTA TTATGTTTTT 3240

TCTTGTGTGT GTGTGTGTGT GTTTGTGTGT GTGCGGTTAT TTTGTATCCG TTTGCTCCCG 3300

CCCCTGCCCC CCATCACCCG AGGAGAAAGT AGAATAAGAC ACATACGATT GTTGTTTTTG 3360

TTATCCTTAA AAGGAAGAGA GACCAAAAAA AAAAAAAAAA AA 3402

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 915 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "protein"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Thr Val Thr Val Asp Leu Phe Asn His Ala Lys Pro Ser Asn Asn
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Glu Gly Arg Val Trp Ser Val Asp Ala Ala Thr Phe Asn Glu Val Pro
            20                  25                  30

Glu Ala Gln Arg Val Leu Ala Asp Ser Gln Phe Tyr Leu Ala Tyr Thr
        35                  40                  45

Met Lys Arg Arg His Val Leu Arg Val Val Lys Arg Ser Asn Leu Leu
    50                  55                  60

Lys Gly Thr Val Arg Ala His Ser Lys Pro Ile His Ala Val Lys Phe
65                  70                  75                  80

Val Asn Tyr Arg Ser Asn Val Ala Ala Ser Ala Gly Lys Gly Glu Phe
                85                  90                  95

Phe Val Trp Val Val Thr Asp Glu Thr Asp Ala Ser Asn Gly Lys Pro
            100                 105                 110

Asp Leu Ala Ala Arg Leu Thr Val Lys Val Tyr Phe Lys Leu Gln Asp
        115                 120                 125

Pro Val Thr Ile Pro Cys Phe Ser Phe Phe Ile Asn Ala Glu Ser Gln
    130                 135                 140

Arg Pro Asp Leu Leu Val Leu Tyr Glu Thr Gln Ala Ala Ile Leu Asp
145                 150                 155                 160

Ser Ser Ser Leu Ile Glu Arg Phe Asp Val Glu Ser Leu Glu Ala Thr
                165                 170                 175

Leu Gln Arg Asn Cys Thr Thr Leu Arg Thr Leu Thr Gln Pro Val Ser
            180                 185                 190

Glu Asn Ser Leu Cys Ser Val Gly Ser Gly Gly Trp Phe Thr Phe Thr
        195                 200                 205

Thr Glu Pro Thr Met Val Ala Ala Cys Thr Leu Arg Asn Arg Ser Thr
    210                 215                 220

Pro Ser Trp Ala Cys Cys Glu Gly Glu Pro Val Lys Ala Leu His Leu
225                 230                 235                 240

Leu Asp Ala Thr Val Glu Glu Asn Val Ser Val Leu Val Ala Ala Ser
                245                 250                 255

Thr Lys Gly Val Tyr Gln Trp Leu Leu Thr Gly Val Ala Glu Pro Asn
            260                 265                 270

Leu Leu Arg Lys Phe Val Ile Asp Gly Ser Ile Val Ala Met Glu Ser
        275                 280                 285

Ser Arg Glu Thr Phe Ala Val Phe Asp Asp Arg Lys Gln Leu Ala Leu
    290                 295                 300

Val Asn Met His Ser Pro His Asn Phe Thr Cys Thr His Tyr Met Met
305                 310                 315                 320
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Pro Cys Gln Val Gln Arg Asn Gly Phe Cys Phe Asn Arg Thr Ala Asp
325 330 335

Gly Ser Cys Val Leu Ala Asp Met Ser Asn Arg Leu Thr Ile Phe His
340 345 350

Leu Arg Cys Ser Arg Arg Glu Glu Gln Gln Pro Gly Gln Lys Thr Ser
355 360 365

Val Val Ala Thr Ala Lys Pro Gly Cys Val Ser Ser Gly Thr Asp Ala
370 375 380

Ala Ser Ser Ser His Thr Asn Thr Thr Ser Ala Ala Ala Ala Ser Pro
385 390 395 400

Ala Ser Pro Pro Val Ser Ala Pro Ala Lys Ala Ala Ala Pro Pro Ala
405 410 415

Ala Ala Arg Ser Ala Glu Pro His Val Gly Ser Lys Ile Ile Ala Asn
420 425 430

Leu Val Asn Gln Leu Gly Ile Asn Val Thr Gln Arg Ser Val Val Ser
435 440 445

Thr Gly Ala Pro Ala Thr Thr Arg Ser Thr Ala Val Thr Ser Thr Thr
450 455 460

Thr Ala Pro Gln Arg Thr Ser Pro Tyr Gly His Asn Gly Arg Pro Val
465 470 475 480

Thr Ala Gly Leu Val Ala Ala Asn Ser Gly Ala Ser Ala Ala Ser Ser
485 490 495

Pro Thr Ala Ala Ala Lys Pro Thr Gly Glu Glu Lys Ala Ser Ala Ala
500 505 510

Cys Glu Thr Ser Ser Val Ala Ile Asn Ala Thr Arg Pro Ala Leu His
515 520 525

Asn Ala Ser Leu Pro Gln Ala Pro Thr Asp Gly Val Leu Ala Ala Ala
530 535 540

Val Tyr Gln Ser Glu Gly Glu Val His Gln Ser Leu Glu Arg Leu Glu
545 550 555 560

Ser Val Ile Thr Asn Thr Ser Arg Val Leu Lys Leu Leu Pro Asp Thr
565 570 575

Ile Arg Arg Asp His Glu Gln Leu Leu Asn Leu Gly Leu Glu Ala Gln
580 585 590

Met Thr Glu Leu Gln Gln Ser Arg Pro Thr Pro Gln Thr Gln Pro Arg
595 600 605

Asp Thr Ser Ser Ala Lys Ser Ser Val Phe Glu Thr Tyr Thr Leu Val
610 615 620

Leu Ile Ala Asp Ser Leu Ser Arg Asn Ile Thr Lys Gly Val Lys Arg
625 630 635 640

Gly Val Asn Glu Ala Ile Met Leu His Leu Asp His Glu Val Arg His
645 650 655

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Ala Ile Gly Asn Arg Leu Arg Gln Thr Gln Lys Asn Ile Ile Lys Ser
                660                 665                 670

Arg Leu Asp Glu Ala Leu Lys Glu Ser Thr Thr Gln Phe Thr Ala Gln
        675                 680                 685

Leu Thr Gln Thr Val Glu Asn Leu Val Lys Arg Glu Leu Ala Glu Val
    690                 695                 700

Leu Gly Ser Ile Asn Gly Ser Leu Thr Ser Leu Val Lys Glu Asn Ala
705                 710                 715                 720

Ser Leu Lys Lys Glu Leu Asn Ser Ile Met Ser Ser Gly Val Leu Asp
                725                 730                 735

Glu Met Arg Arg Met Arg Glu Glu Leu Cys Thr Leu Arg Glu Ser Val
                740                 745                 750

Ala Lys Arg Lys Ala Thr Met Pro Asp Ser Ser Leu His Ala Thr Ser
        755                 760                 765

Ser Phe Gln Gly Arg Arg Ser Ala Pro Glu Thr Ile Leu Ala Thr Ala
    770                 775                 780

Leu Ser Met Val Arg Glu Gln Gln Tyr Arg Gln Gly Leu Glu Val Met
785                 790                 795                 800

Leu Met Ala Gln Gln Pro Ser Leu Leu Leu Arg Phe Leu Ser Ile Leu
                805                 810                 815

Thr Arg Glu Asn Glu Asn Ala Tyr Ser Glu Leu Ile Glu Asn Val Glu
                820                 825                 830

Thr Pro Asn Asp Val Trp Cys Ser Val Leu Leu Gln Leu Ile Glu Ala
        835                 840                 845

Ala Ala Thr Glu Ala Glu Lys Glu Val Val Val Gly Val Ala Ile Asp
    850                 855                 860

Ile Leu Ser Glu Arg Asp Gln Ile Ala Gln Asn Gly Ala Leu Gly Ser
865                 870                 875                 880

Lys Leu Thr Thr Ala Met Arg Ala Phe Glu Arg Gln Ala Arg Ser Glu
                885                 890                 895

Thr Thr Ser Arg Ser Phe Leu Gln Cys Leu Lys Asn Leu Ile Lys Leu
                900                 905                 910

Leu Gln Ser
        915
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "phage DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTGGCGACG ACTCCTGGAG CCCG 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "phage DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TTGACACCAG ACCAACTGGT AATG 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCGGGCACTG ACGCGGCG 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "phage lambda gt10 DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTTATGAGTA TTTCTTCCAG GGTA 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AACGCTATTA TTAGAACAGT T 21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCAGCAGCG GCAGAAGT 18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAGCCGACGG TAGCTGCGTC CT 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACATAATGGC CTCGTTCACA C 21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACTCGCTGC AGATCGATTT TTTTTTTTTT TTTT 34

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGAAGAGACC ATGAACAACT T 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACTCGCTGC AGATCGAT 18

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CLAIMS

1. A synthetic or isolated nucleic acid fragment which comprises a nucleotide sequence that is identical, complementary, antisense or equivalent to a first sequence starting at nucleotide 1232 and ending at nucleotide 1825 of SEQ ID NO:1.

2. The nucleic acid fragment according to claim 1, wherein said nucleotide sequence is identical, complementary, antisense or equivalent to a second sequence starting at nucleotide 1232 and ending at nucleotide 2207 of SEQ ID NO:1.

3. The nucleic acid fragment according to claim 1, wherein an at least 30 nucleotide segment of said nucleotide sequence is at least 50% homologous with a correspondingly long segment of the sequence identified in SEQ ID NO:1.

4. The nucleic acid fragment according to claim 2, wherein an at least 30 nucleotide segment of said nucleotide sequence is at least 50% homologous with a correspondingly long segment of the sequence identified in SEQ ID NO:1.

5. A probe for identifying Trypanosoma cruzi, said probe comprising a nucleotide sequence that is hybridizable to at least a segment of a nucleic acid according to claim 1.

6. The probe according to claim 5, wherein said probe comprises 5 to 100 nucleotides.

7. The probe according to claim 5, wherein said probe comprises 8 to 50 nucleotides.

8. A primer for amplifying a nucleotide sequence, said primer comprising a nucleotide sequence that allows hybridization to at least a segment of a nucleic acid according to claim 1.

9. The primer according to claim 8, wherein said nucleotide sequence comprises at least five nucleotides.

10. The primer according to claim 9, wherein said nucleotide sequence is selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:12.

11. A reagent for detecting or identifying Trypanosoma cruzi in a biological sample, said reagent comprising at least one of a capture probe and a detection probe, wherein said capture probe and said detection probe each comprise a nucleotide sequence that is hybridizable to at least a segment of a nucleic acid according to claim 1, and wherein said capture probe and said detection probe, if they are both present, have nucleotide sequences that are at least partially different from one another.

12. The reagent according to claim 11, wherein said capture probe is attached to a solid support.

13. The reagent according to claim 12, wherein said capture probe is directly attached to said solid support.

14. The reagent according to claim 12, wherein said capture probe is indirectly attached to said solid support.

15. The reagent according to claim 11, wherein said detection probe is labelled by a marker selected from the

group consisting of radioactive isotopes, enzymes capable of hydrolyzing a chromogenic, fluorigenic or luminescent substrate, chromophoric chemical compounds, fluorigenic compounds, luminescent compounds, nucleotide base analogs, and biotin.

16. The reagent according to claim 15, wherein said enzymes are selected from the group consisting of peroxidase and alkaline phosphatase.

17. The reagent according to claim 11, comprising at least one primer comprising a nucleotide sequence that allows hybridization to at least a segment of a nucleic acid which comprises a nucleotide sequence that is identical, complementary, antisense or equivalent to a first sequence starting at nucleotide 1232 and ending at nucleotide 1825 of SEQ ID NO:1.

18. A method for detection and/or identification of Trypanosoma cruzi in a biological sample, comprising exposing to at least one probe according to claim 5 denatured DNA extracted from Trypanosoma cruzi or DNA obtained by reverse transcription of RNA extracted from Trypanosoma cruzi; and detecting hybridization of said probe.

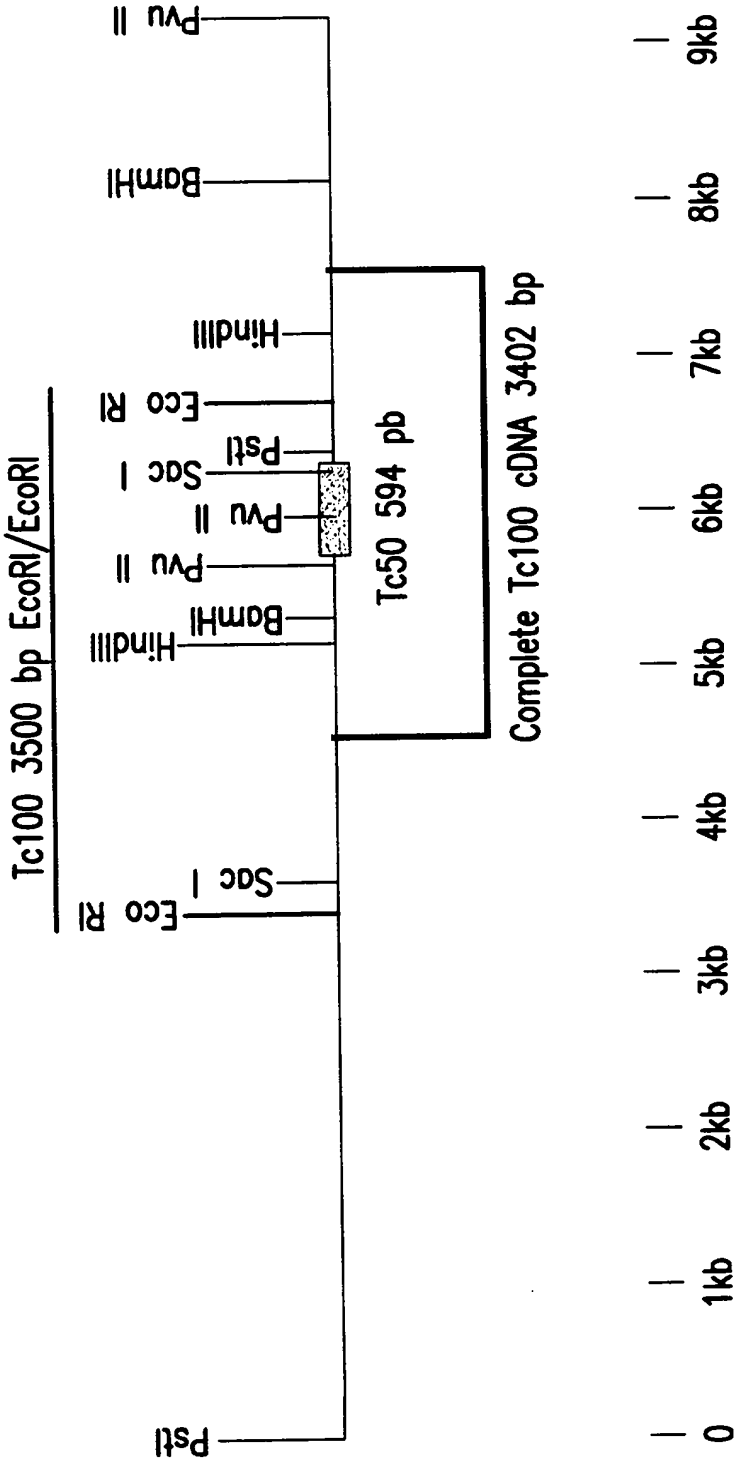
19. A method for detection and/or identification of Trypanosoma cruzi in a biological sample, comprising exposing extracted RNA from Trypanosoma cruzi to at least one probe according to claim 5; hybridizing said probe with said RNA; and detecting said hybridization.

20. The method according to claim 18, wherein before said DNA is exposed to said probe, said DNA is

amplified in the presence of an enzymatic system with at least one primer, wherein said primer comprises a nucleotide sequence that is hybridizable to a nucleic acid sequence that is identical, complementary, antisense or equivalent to a sequence identified in SEQ ID NO:1.

ABSTRACT OF THE DISCLOSURE

The nucleotide sequence of Tc100, a gene encoding PTc100, a new Trypanosoma antigen, and the amino acid sequence of PTc100 are described. Tc100 and PTc100, or fragments thereof, modified or otherwise, can be used directly or indirectly for the detection of Trypanosoma cruzi, or for the monitoring of the infection generated by Trypanosoma cruzi, in man or in animals.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Sac I
Tc100 3500 bp EcoRI/EcoRI
HindIII
BamHI
Pvu II
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HindIII
BamHI
Pvu II
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FIG.1

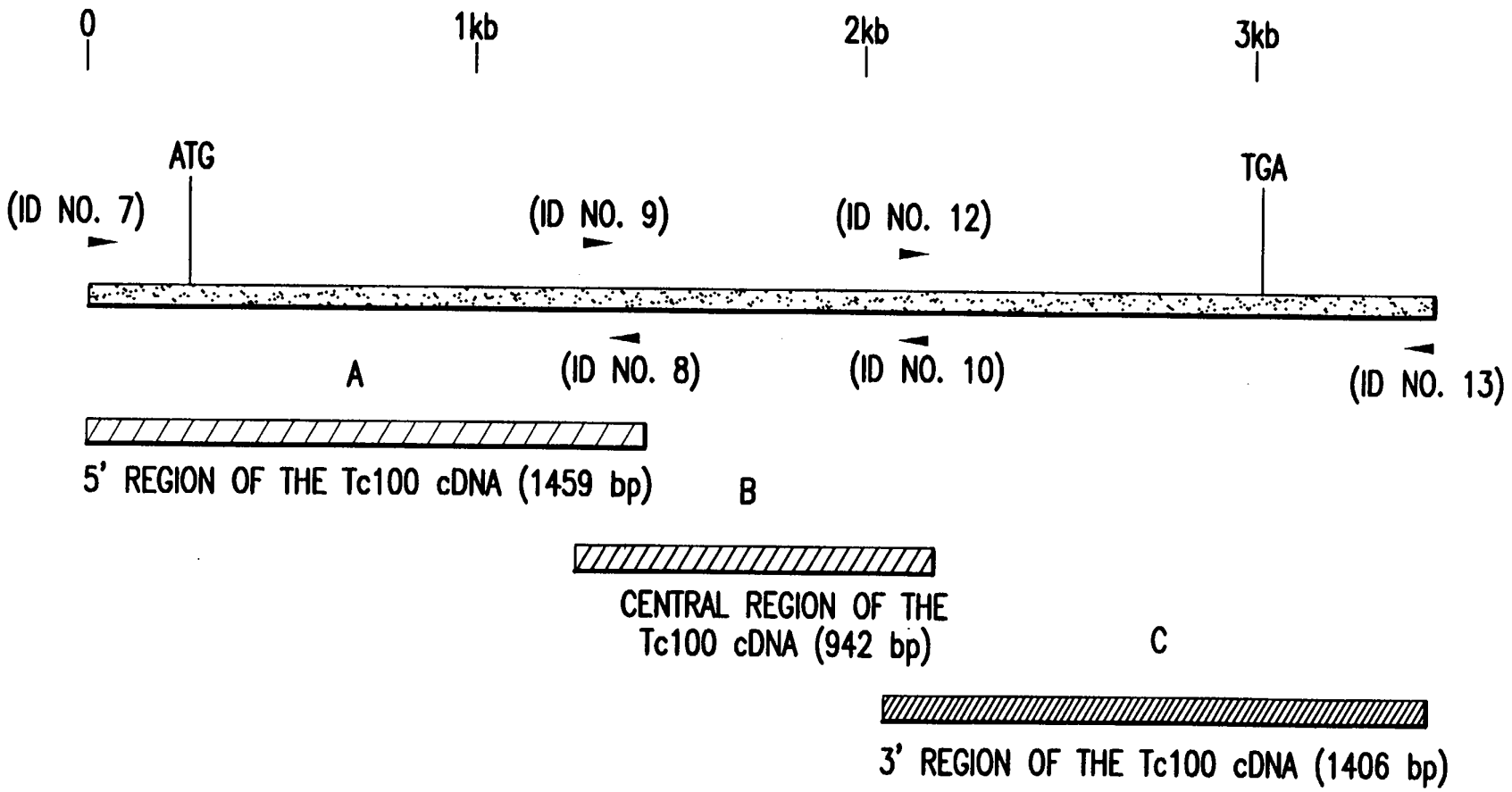
0
1kb
2kb
3kb
ATG
TGA
(ID NO. 7)
(ID NO. 9)
(ID NO. 12)
(ID NO. 8)
(ID NO. 10)
(ID NO. 13)
A
5' REGION OF THE Tc100 cDNA (1459 bp)
B
CENTRAL REGION OF THE
Tc100 cDNA (942 bp)
C
3' REGION OF THE Tc100 cDNA (1406 bp)

FIG.2

Docket No.: WPB 36400

APPLICATION FOR UNITED STATES PATENT DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: NEW TRYPANOSOMA CRUZI ANTIGEN, GENE ENCODING THEREFOR AND METHODS OF DETECTING AND TREATING CHAGAS DISEASE

described and claimed in the specification:

Check one

*a. ☐ attached hereto.

b. ☒ filed on June 7, 1995 as Application No. 08/480,917 and amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) and/or United States provisional application(s) filed within one year prior to this application are hereby claimed:

French Patent Application No. 94 10132, filed August 12, 1994

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to this application, or (b) before the filing date of the above-named foreign priority application(s) and/or United States provisional application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

**James A. Oliff, Reg. No. 27,075; William P. Berridge, Reg. No. 30,024;
Kirk M. Hudson, Reg. No. 27,562; Thomas J. Pardini, Reg. No. 30,411;
Edward P. Walker, Reg. No. 31,450; Robert A. Miller, Reg. No. 32,771 and
Mario A. Costantino, Reg. No. 33,565.**

ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, PLC, P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 ***Typewritten Full Name of First or Sole Inventor***

Glaucia		PARANHOS-BACCALA
Given Name	Middle Initial	Family Name

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3 **Date of Signature:

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Month	Day	Year

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**Note to Inventor: Please sign name exactly as it appears above and insert actual date of signing.

IF THERE IS MORE THAN ONE INVENTOR USE PAGE 2 AND PLACE AN "X" HERE ☒

10/96

(Discard this page in a sole inventor application)

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Inventor's Signature

Date of Signature

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Citizenship

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Typewritten Full Name of Joint Inventor: Given Name — Middle Initial — Family Name

2 Inventor's Signature

3 Date of Signature

Residence: City — State or Province — Country

Citizenship

Post Office Address (Insert complete mailing address, including country)

1 Typewritten Full Name of Joint Inventor: Given Name — Middle Initial — Family Name

2 Inventor's Signature

3 Date of Signature

Residence: City — State or Province — Country

Citizenship

Post Office Address (Insert complete mailing address, including country)

Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

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